Vasoregression in incipient diabetic retinopathy

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CHAPTER 5

Pericyte migration: A novel mechanism of pericyte loss in experimental diabetic retinopathy

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Abstract

Objective: The mechanism underlying pericyte (PC) loss during incipient diabetic retinopathy remains controversial. Hyperglycemia induces angiopoietin-2 (Ang-2) transcription, which modulates capillary PC coverage. In this study, we assessed loss of PC subgroups and the contribution of Ang-2 to PC migration.

Research Design and Methods: Numbers of total PCs and their subgroups were quantified in retinal digest preparations of spontaneous diabetic XLacZ mice. PCs were divided into subgroups according to their localization, their position relative to adjacent endothelial cells and the expression of LacZ. The contribution of Ang-2 to PC migration was assessed in Ang-2 overexpressing (mOpsinhAng2) and deficient (Ang2LacZ) mice.

Results: Pericyte numbers were reduced by 16 % ($p < 0.01$) in XLacZ mice after 6 months of diabetes. Reduction of pericytes was restricted to pericytes on straight capillaries (relative reduction 27%, $p < 0.05$) and predominantly observed in LacZ positive PCs (-20 %, $p < 0.01$). Hyperglycemia increased the numbers of migrating PCs (+69%; $p < 0.05$), of which the relative increase due to diabetes was exclusively in LacZ negative PCs, indicating reduced adherence to the capillaries (+176%; $p < 0.01$). Overexpression of Ang-2 in non-diabetic retinas mimicked diabetic PC migration of wild-type animals (+78%; $p < 0.01$). Ang-2 deficient mice completely lacked hyperglycemia-induced increase in pericyte migration compared to wild-type littermates.

Conclusion: Diabetic pericyte loss is the result of pericyte migration and this process is modulated by the Ang-Tie system.

Introduction

Pericytes (PC) are heterogeneous with regard to their origin, distribution, phenotype and function [195, 196]. Resident retinal pericytes derive from mesoderm and neural crest during development. A bone marrow origin of pericytes and transdifferentiation of endothelial cells into pericytes have also been demonstrated during postnatal vascular repair and in adult angiogenesis [197-199]. Furthermore, pericytes can transdifferentiate into macrophage- and fibroblast-like cells [200, 201]. Pericytes are the capillary counterparts to smooth muscle cells (SMC) on arterioles. In contrast to SMC, they are completely embedded within the capillary basement membrane and extend processes, varying in length, arrangement and form, indicating their mobility in functional blood vessels [13, 14, 183, 202]. One remarkable common feature of PCs and SMCs is their contractile phenotype [203]. However, the key molecules actin and myosin for vessel constriction are unequally distributed within the pericyte population [204-206], leading to differences in their contractive potential, depending on their localization within the capillary tree [203]. Pericytes can control endothelial cell proliferation and angiogenesis, both under physiological and pathological conditions [126, 207-212]. Smooth muscle actin (SMA), desmin, proteoglycan NG2, platelet-derived growth factor-receptor beta (PDGFB-R), the aminopeptidase N and the regulator of G-signaling 5 (RGS-5) are common pericyte markers, but none of them is sufficient to recognize every pericyte [169, 177, 206, 210, 213-216]. Another experimental tool for studying pericytes is the XLacZ mouse. In this mouse, LacZ is expressed in vascular smooth muscle cells and pericytes at distinct levels of the vascular tree, but cell proliferation and migration are associated with transgene down-regulation. To our own observations LacZ is expressed only in a subset of retinal pericytes. The diversity of origin, differences in functional capacity and the lack of a pan-pericyte marker suggest that the pericyte population is not uniform in a given organ and hence, the response of retinal pericytes on chronic hyperglycemia may differ in certain subpopulations.

Diabetic retinopathy (DR) is morphologically characterized by pathological changes in the retinal capillaries. The primary and predominant characteristics are the loss of pericytes and the progressive occlusion of capillaries [13, 179]. It is presumed that prevention of the earliest events in the pathogenesis of DR, such as pericyte loss, will prevent the subsequent development of DR. The underlying mechanisms responsible for pericyte loss in DR are complex and not completely elucidated.

Apoptosis and destructive signaling pathways within pericytes are generally discussed to be the reasons for retinal pericyte loss under hyperglycemic conditions [116, 117], but growing evidence suggests that pericytes are actively depleted by alternative mechanisms. Pericyte coverage of capillaries is modulated by a variety of growth factors systems, such as angiopoietins and their tyrosine kinase receptor Tie-2. Angiopoietin-2 (Ang-2) is expressed in the retina and is upregulated in the diabetic retina, prior to pericyte loss. We recently demonstrated that a 50 % reduction of Ang-2 gene dose prevents pericyte loss in the diabetic retina, suggesting an important role of Ang-2/Tie-2 system in diabetic pericyte loss. Constitutive overexpression of Ang-2 in photoreceptor cells reduces pericyte coverage in the deep capillary layers of the retina and injection of recombinant Ang-2 into the vitreous of non-diabetic rats induced a dose-dependent pericyte loss within days, indicating the importance of Ang-2 in the reduction of pericyte coverage [28, 184]. Increased levels of Ang-2 in diabetic animals and in vitreous fluid of patients with proliferative DR support the role of Ang-2/Tie-2 system in the pathogenesis of DR [140, 190], but the underlying mechanisms remain to be investigated.

In the light of published evidence for the heterogeneity of pericytes and the association of pericyte loss with the Ang- 2/Tie-2 system, we hypothesized that diabetic pericyte loss is the result of other mechanisms than apoptosis in situ and that factors such as Ang-2 may actively modulate this process.

In this study, we used transgenic mouse models for quantification of pericyte loss in experimental DR to investigate the response of retinal pericytes to chronic hyperglycemia

Materials and Methods

Animals

All experiments in this study were performed according to the guidelines of the statement of animal experimentation issued by the Association for Research in Vision and Ophthalmology (ARVO) and were approved by the Institutional Animal Care and Use Committee. Animals were housed in groups in cages with free access to standard chow and water under a 12 hr light and 12 hr dark rhythm.

Spontaneous diabetic Ins2Akita heterozygous mice, purchased from Jackson Laboratory (Charles River Laboratories, Sulzfeld, Germany) were bred with homozygous XLacZ mice, which express the reporter gene LacZ under the control of a smooth muscle and pericyte specific promoter [169] to generate spontaneous diabetic heterozygous XLacZ mice (Ins2Akita+/-XLacZ+/-). Age matched non-diabetic heterozygous XLacZ mice (Ins2Akita-/-XLacZ+/-) served as control.

Genotype was determined by PCR 4 weeks after birth as described previously [217]. Only male mice were used in this study as the diabetes onset is earlier and hyperglycemia is more severe compared with female mice. Glucose levels and body weight were monitored consecutively every other week, and glycated haemoglobin concentration was determined by affinity chromatography at the end of the study (MicromatIITM; Bio-Rad Laboratories GmbH, Munich, Germany). Insulin was occasionally given to individual diabetic mice to prevent critical weight loss. After six months of experimental diabetes, eyes were enucleated under deep anesthesia and immediately frozen at -80°C for further analysis.

To study the impact of Ang-2 on pericyte migration, retinas of transgenic mOpsinAng2 and Ang2LacZ mice were analyzed. mOpsinhAng2 mice overexpress human Ang-2 in the photoreceptor layer of the retina. Ang2LacZ mice carry a targeting vector that replaces part of the coding region of Ang-2 with the LacZ gene encoding beta- galactosidase with the intention of creating a null allele of Ang-2. Heterozygous LacZ knock-in results in a 50% reduction of functional Ang-2 gene dose. Generation and genotyping of mOpsinAng2 and Ang2LacZ mice have been described previously [162, 184]. Wild-type litters served as controls. Diabetes was induced by a single injection of streptozotocin (STZ; purchased from Roche, Mannheim, Germany; 150 mg/kg i.p.) in randomly selected animals of transgenic and wild-type mice. STZ-injected animals were considered diabetic when blood glucose levels reached stable levels over 250 mg/dl. Diabetic and non-diabetic mice were

sacrificed 6 months after diabetes induction and eyes were collected under deep anesthesia and immediately frozen at - 80°C.

LacZ staining

Eyes of XLacZ mice were stained for beta-galactosidase activity according to an established protocol [28]. In brief, eyes were first fixed (phosphate buffer 100 mM, pH 7.3 containing 1.5% (v/v) formaldehyde/0.2% (v/v) glutaraldehyde) and then incubated in a staining solution containing 0.1% X-gal (Roche Diagnostics GmbH, Mannheim, Germany), 5 mM K4Fe(CN)6, and 5 mM K3Fe(CN)6 over night at 37 degree. After washing in PBS, retinas were isolated and subjected to the retinal digestion procedure (see below).

Immunofluorescence analysis

To examine the relationship of migrating pericytes with the underlying endothelium, retinal whole mounts fixed in 4% paraformaldehyd were permeabilized in 1% BSA and 0.5% Triton X-100 at room temperature for 1 h and incubated with a rabbit anti NG2 chondroitin sulphate proteoglycan polyclonal antibody (Chemicon International, Hampshire, UK, dilution 1:200) and TRITC-labeled isolectin B4 from Bandeiraea simplicifolia (Sigma-Aldrich, München, Germany; dilution 1:50) overnight at 4°C. FITC-conjugated swine anti rabbit IgG (DakoCytomation GmbH, Hamburg, Germany, dilution 1:20) was used for the detection of NG2 primary antibody. Photos were taken using a confocal microscope (Leica TCS SP2 Confocal Microscope, Leica).

Retinal digest preparations

Vascular preparations of whole-mount retinas of XLacZ, mOpsinhAng2 and Ang2LacZ mice were performed using a trypsin digestion technique as previously described [8, 33]. Briefly, retinas were digested in a solution containing 3 % trypsin resolved in 0.2 mol/l Tris-HCl solution at 37 °C for 3 h. The retinal vasculature was isolated by dropping water and dried on objective slides. Subsequently, retinal digest samples were stained with periodic acid Schiff base [218] and hematoxylin.

Morphological quantification

To determine numbers and morphologies of retinal pericytes, retinal digest preparations of spontaneous diabetic and non-diabetic XLacZ mice (n=7-8) were analyzed. First, total numbers of pericytes were counted in 10 randomly selected areas (magnification 400x) in

the middle sector of the retina, using an image analyzing system (CUE-2; Olympus Opticals, Hamburg, Germany). Both areas, close to the optic nerve and to the external border of the retina were excluded from analysis. Numbers were standardized relative to the capillary density (numbers of cells per mm2 of capillary area). Subsequently, according to their localization within the capillary tree and their position relative to adjacent endothelial cells, pericytes were divided into three subgroups. Pericytes were located either at capillary branches (B-PC) or on straight capillaries (S-PC) with broad contact to the underlying endothelium, or on straight capillaries with less area of contact to the microvasculature (M-PC). Pericytes with triangular nuclei, migrating from capillaries into the extravascular intestitium were defined as M-PCs as at least one lateral side of the triangular nuclei was longer than the basis in contact to the capillary. Migrating pericytes usually extended processes to neighboring capillaries. Finally, according to the LacZ staining, each group described above was divided into two subgroups, e.g. B-PC into B-PC+ (i.e. pericytes at branches stained positive for LacZ) and B-PC- (i.e. LacZ negative pericytes at branches). The numbers of pericytes in 10 randomly selected fields of the retina were standardized relative to the capillary density (number of cells per mm2 of capillary area). All samples were evaluated in a masked fashion.

To examine the influence of Ang-2/Tie- 2 system on pericyte loss in DR, we quantified the numbers of migrating pericytes (M-PCs) in retinas of non-diabetic and diabetic mOpsinhAng2 (n= 7-8 animals per group) and Ang2LacZ mice (n= 6) in comparison to non-diabetic and diabetic wild-type litters (n= 8).

Statistical analysis

Quantitative data are given as mean ± SD. Student t-test was used to make comparison between groups. A value of $p < 0.05$ was considered statistically significant.

Results

Metabolic data of XLacZ mice, mOpsinhAng2 and Ang2LacZ mice

Diabetic XLacZ mice developed elevated blood glucose levels after the fourth postnatal week. Two weeks later, most diabetic males reached stable blood glucose levels over 500 mg/dl. Final blood glucose was significantly elevated in diabetic XLacZ mice (non-diabetic vs. diabetic XLacZ mice: 147.1 ± 17.9 mg/dl and 599.8 ± 0.7 mg/dl; $p < 0.001$) compared with non-diabetic XLacZ mice. At the end of the study diabetic XLacZ mice showed a 32% reduced body weight compared with non-diabetic XLacZ mice (non-diabetic vs. diabetic XLacZ mice: 35.9±4.7g and 24.3±3.3g;$p<0.001$). Glycated haemoglobin levels were 2.2 fold increased in diabetic XLacZ mice compared with non-diabetic mice (non-diabetic vs. diabetic XLacZ mice: 5.8 ± 0.6% and 12.8 ± 1.8%; $p < 0.01$, Tab. 1).

After 6 months of hyperglycemia, diabetic mOpsinhAng2 mice showed significantly elevated levels of blood glucose (non-diabetic vs. diabetic mOpsinhAng2 mice: 184.6 ± 19.0 mg/dl and 575.0 ± 40.9 mg/dl, $p < 0.001$) and Hb1Ac (non-diabetic vs. diabetic mOpsinhAng2 mice: 5.7 ± 0.5% and 10.7 ± 2.1%, $p < 0.001$) compared with their non-diabetic litters. Body weights of diabetic mOpsinhAng2 mice were significantly reduced (non-diabetic vs. diabetic mOpsinhAng2 mice: 34.5 ± 4.6 g and 28.0 ± 3.9, $p < 0.01$, Tab. 1).

In diabetic Ang2LacZ mice final blood glucose levels and Hb1Ac were also significantly increased (non-diabetic vs. diabetic Ang2LacZ: 165.5 ± 26.1 mg/dl and 582.7 ± 42.5 mg/dl blood glucose, $p < 0.001$ and 5.4 ± 0.1% and 11.1 ± 1.9% HbA1c, $p < 0.01$) and chronic hyperglycemia led to weight loss of 29% in diabetic Ang2LacZ mice (non-diabetic vs. diabetic Ang2LacZ: 34.2±3.5g and 24.3±4.5g,$p<0.05$,Tab. 1)

	Nondiabetic XLacZ (n=11)	Diabetic XLacZ (n=8)
Glucose level (mg/dl)	147.1 ± 17.9	599.8 ± 0.7 *
body weight (g)	35.9 ± 4.7	24.3 ± 3.3 *
HbA1c (%)	5.8 ± 0.6	12.8 ± 1.8 *
	Nondiabetic mOpsinhAng2 (n=7)	Diabetic mOpsinhAng2 (n=8)
Glucose level (mg/dl)	184.6 ± 19.0	575.0 ± 40.9 †
body weight (g)	34.5 ± 4.6	28.0 ± 3.9 *
HbA1c (%)	5.7 ± 0.5	10.7 ± 2.1 †
	Nondiabetic Ang2LacZ (n=6)	Diabetic Ang2LacZ (n=6)
Glucose level (mg/dl)	165.5 ± 26.1	582.7 ± 42.5 §
body weight (g)	34.2 ± 3.5	24.3 ± 4.5 *
HbA1c (%)	5.4 ± 0.1	11.1 ± 1.9 †

Table 1: Metabolic data of non-diabetic and diabetic mice. Diabetic XLacZ ($p < 0.001$), mOpsinhAng2 (* $p < 0.01$, † $p < 0.001$) and Ang2LacZ mice (* $p < 0.05$, † $p < 0.01$, § $p < 0.001$) were compared to their non-diabetic litters.*

Differential pericyte loss in pericyte subpopulation

Pericytes show variable phenotypes. As demonstrated in Fig. 1 A, pericytes were located either at capillary branches or on straight capillaries. PCs on straight capillaries showed small, round or elongated nuclei. Notably, one type of PCs on straight capillaries appeared to have less contact to the endothelium. Fig. 1 B-G illustrates PC migration continuum process from broad to less contact to the underlying endothelium, which we frequently found at different stages in our studies. Pericytes in Fig. 1 D-G were defined as migrating PCs. To examine the relationship between pericyte and the adjacent endothelium during the migration process and to exclude artifacts of the digestion procedure, NG2 (green)/ lectin (red) double stainings of retinal whole mounts were performed (Fig. 1 H-J). In contrast to pericytes located on capillaries, migrating pericytes and their extensions to neighboring vessels were solely positive for NG2, suggesting that these structures derive from pericytes and that this pericyte phenotype is neither the consequence of vasoregression nor due to the digestion procedure.

To assess the effect of elevated blood glucose on pericyte loss, we analyzed pericyte coverage in retinal digest preparations of diabetic XLacZ mice after 6 months of experimental hyperglycemia. We found a 16 % reduction of total pericyte numbers after 6 months of hyperglycemia in diabetic XLacZ mice compared with their non-diabetic littermates (non-diabetic vs. diabetic: 2374 ± 257 and 2002 ± 136 per mm2 of capillary area, $p < 0.01$, Fig. 2 A). Analysis of pericyte subgroups revealed that pericytes on vessel branches (B-PCs) were completely unaffected by chronic hyperglycemia (non-diabetic vs. diabetic: 580±157 and 586±139 per mm2 of capillary area, Fig. 2 B). We found that the loss of pericytes in diabetic retinas was limited to pericytes on straight capillaries (S-PCs). Numbers of S-PCs were reduced by 27% (non-diabetic vs. diabetic: 1676 ± 316 and 1218 ± 282 per mm2 of capillary area; $p < 0.05$, Fig. 2 C) and this reduction completely explained the reduction in total pericyte numbers in diabetic animals. Hyperglycemia-induced reduction of pericytes was due to a loss of pericytes located on straight capillaries, accompanied by increased numbers of pericytes detaching preferably from straight parts of retinal capillaries. Hyperglycemia led to increased numbers of pericytes which were involved in morphologically defined migration processes (M-PC). The numbers of M-PCs increased by more than 69 % in diabetic XLacZ retinas after 6 months of hyperglycemia compared with non-diabetic XLacZ retinas (non-diabetic vs. diabetic: 117 ± 38 and 198 ± 79 per mm2 of capillary area; $p < 0.05$, Fig. 2 D).

Furthermore, we analyzed PC loss in different PC subpopulation according to LacZ expression in XLacZ mice. In each subgroup mentioned above, there were LacZ positive and negative pericytes as shown in Fig. 3 A-F.

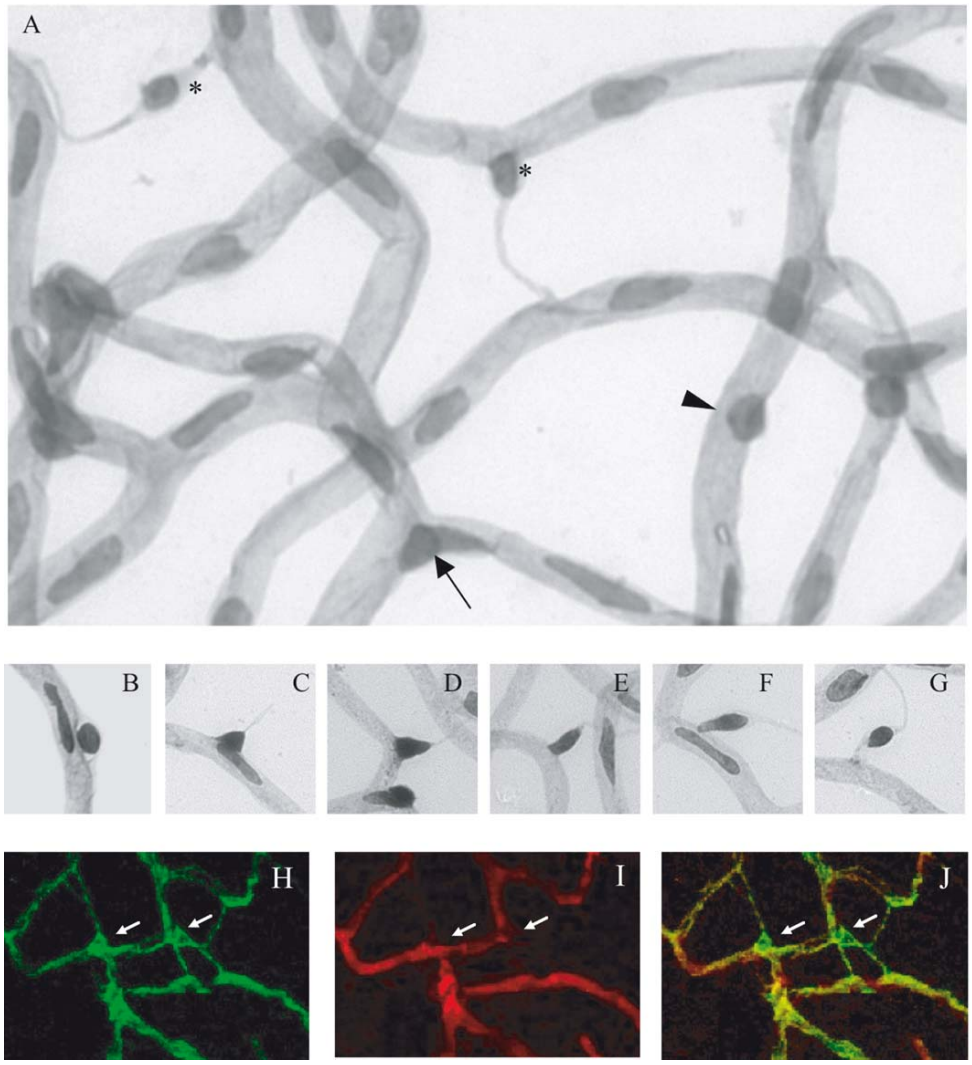

Figure 1: Illustration of pericyte subpopulations according to their location and contact to capillaries (Fig. 1 A) and of pericyte migration process (Fig 1 B-G) in retinal digest preparations. Arrow in A: B-PCs, pericytes at branches; arrowhead in A: S-PCs, pericytes on straight capillaries with broad contact to endothelium; asterisks in A: M-PCs, migrating pericytes on straight capillaries with less contact to endothelium. The continuum of pericyte migration process is depicted in Fig. 1 B-G. According to our morphological definition, pericytes were considered as migrating pericytes (M-PCs) as at least one lateral side of the triangular nuclei was longer than the basis in contact to the capillary (Fig. 1 D-G). Migrating pericytes usually extended processes to neighboring capillaries. Confocal microscopy of NG2 (green)/lectin (red) stained retinal whole mounts identified pericytes (arrows in H-J) and their processes to be dissociated from the underlying endothelium (Fig 1 H-J). H: NG2-FITC staining; I: lectin-TRIC staining; J: merged image of H and I. Original magnification: 400x

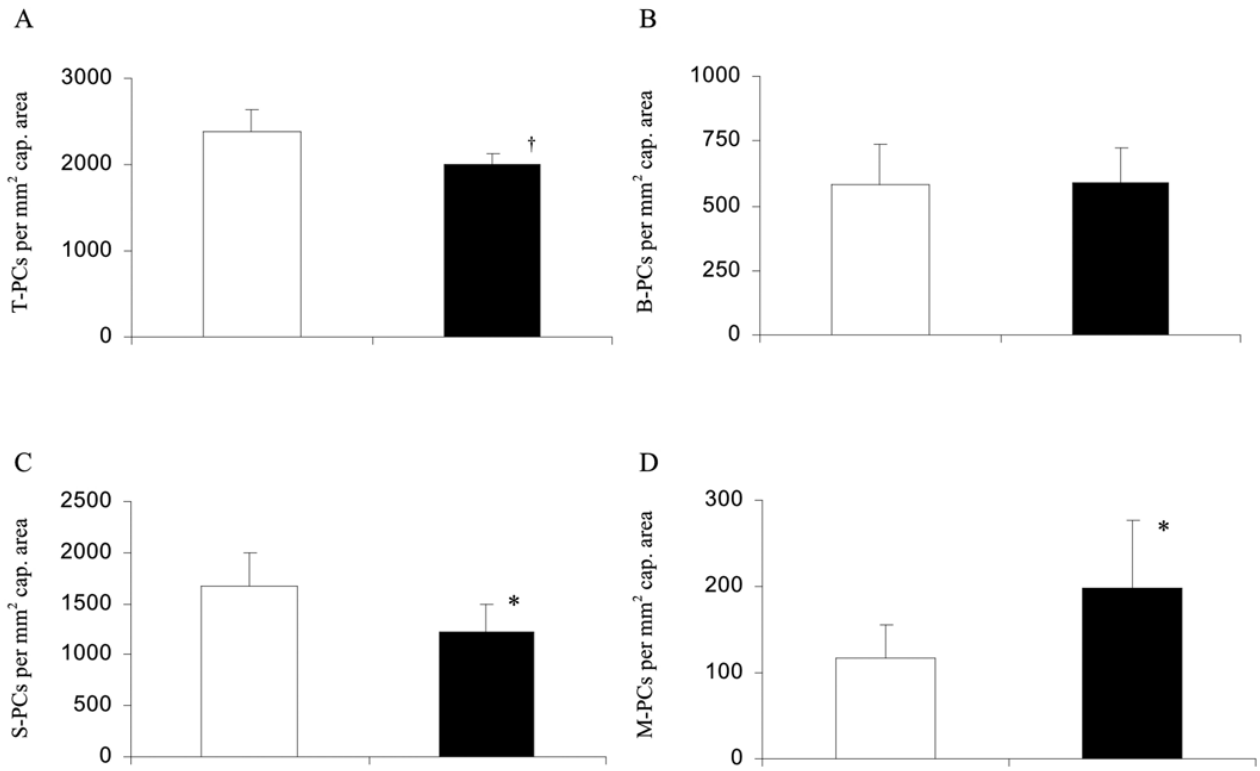

*Figure 2: Quantitation of pericyte subpopulations in non-diabetic and diabetic XLacZ mice. Total numbers of pericytes (T-PC, Fig. 2 A), numbers of pericytes at branches (B-PC, Fig. 2 B), pericytes on straight capillaries with broad contact to endothelium (S-PC, Fig. 2 C) and migrating pericytes (M-PC, Fig. 2 D) in non-diabetic and diabetic XLacZ mice (white bars: non-diabetic and black bars: diabetic) after 6 months of hyperglycemia. * $p < 0.05$, † $p < 0.01$*

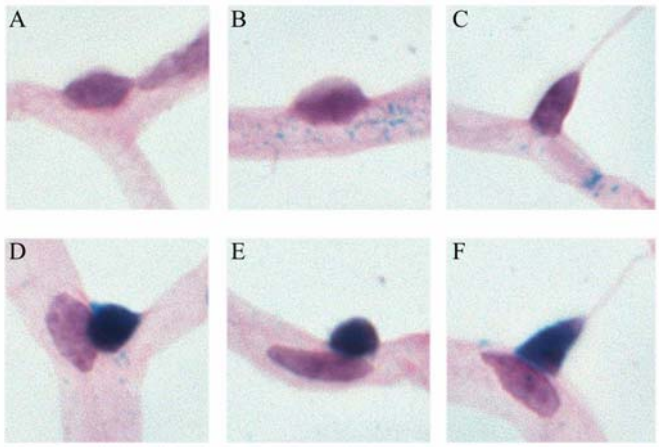

Figure 3: Illustration (Fig. 3 A-F) of pericyte subpopulations considering their location and LacZ expression in XLacZ retinas. Pericytes at branches (Fig. 3 A, D), pericytes on straight capillaries with broad contact to endothelium (Fig. 3 B, E) and migrating pericytes (Fig. 3 C, F) were divided into LacZ positive (e.g. T-PC+, Fig. 3 D-F) and LacZ negative (e.g. T-PC-, Fig. 3 A-C) stained pericytes. Original magnification 400x.

52% of total pericytes were stained LacZ positive, both in non-diabetic and diabetic retinas, but only LacZ positive pericytes were significantly reduced. LacZ positive PCs showed a reduction of 20% (non-diabetic vs. diabetic: 1258 ± 134 and 1008 ± 172 per mm2 of capillary area; $p < 0.01$, Fig. 4 A). Numbers of LacZ negative pericytes were only insignificantly changed (non-diabetic vs. diabetic: 1115 ± 158 and 994 ± 110 per mm2 of capillary area; $p > 0.05$, Fig. 4 A). There was no change in the numbers of LacZ positive and LacZ negative B-PCs (Fig. 4 B). The reduction of S-PCs was due to a significant loss of LacZ positive and LacZ negative pericytes (Fig. 4 C). LacZ positive S-PCs were reduced by 32% (non-diabetic vs. diabetic: 799 ± 152 and 543 ± 181 per mm2 of capillary area; $p < 0.05$) and LacZ negative S-PCs were reduced by 23% in diabetic animals (non-diabetic vs. diabetic: 878 ± 188 and 674 ± 145 per mm2 of capillary area; $p < 0.05$). Increased numbers of M-PCs rose from pericytes which were negative for LacZ expression. LacZ negative M-PCs increased over 2.5 fold (non-diabetic vs. diabetic: 21 ± 10 vs. 79 ± 43 per mm2 of capillary area; $p < 0.01$) in diabetic retinas (Fig. 4 D).

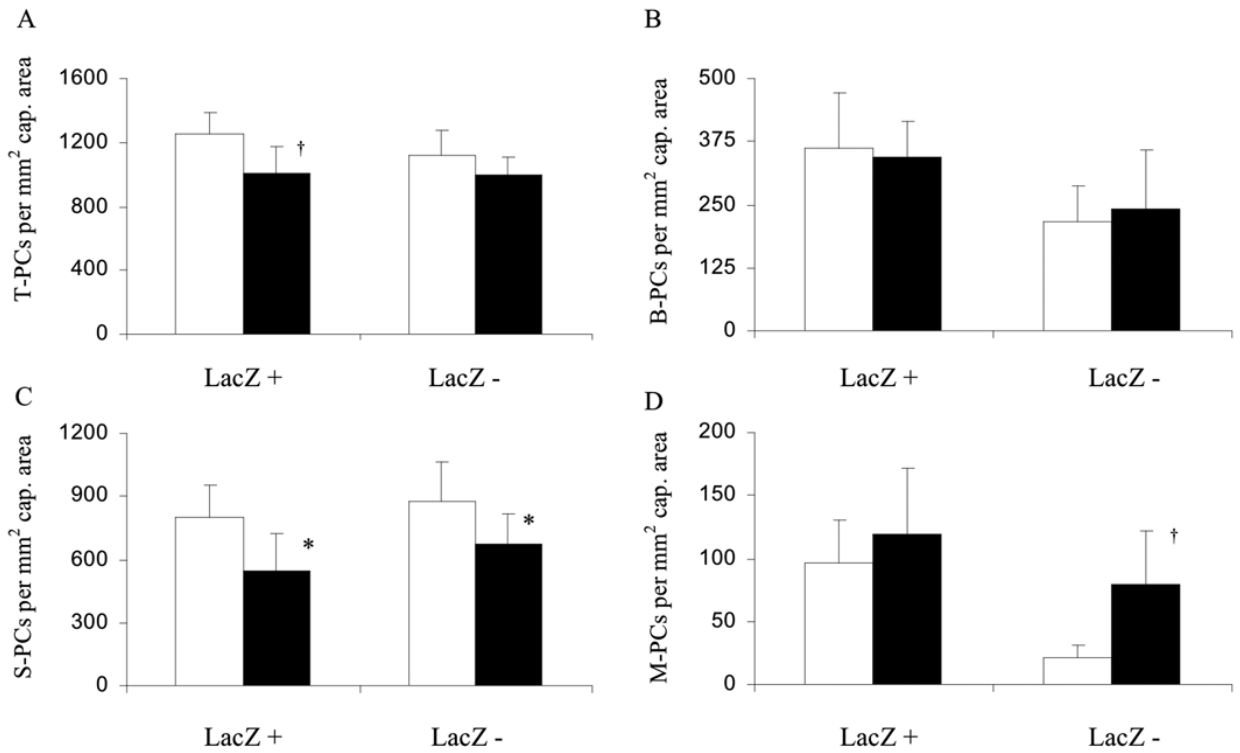

*Figure 4: Quantitation of pericyte subpopulations considering their location and LacZ expression in non-diabetic and diabetic XLacZ retinas. Numbers of total pericytes (T-PC, Fig. 4 A) and numbers of B-PCs (Fig. 4 B), S-PCs (Fig. 4 C) and M-PCs (Fig. 4 D) were quantified after six months of hyperglycemia (white bars: non-diabetic and black bars: diabetic). * $p < 0.05$, † $p < 0.01$*

Influence of Ang-2 modulation on pericyte migration

The Ang-Tie system is crucially involved in pericyte recruitment and attachment. Ang-2 is upregulated by hyperglycemia. Therefore, we examined the effect of Ang-2 changes on pericyte migration (Fig. 5).

As expected, diabetic wild-type (wt) mice showed significantly increased numbers of migrating pericytes up to 70% compared with non-diabetic wild-type mice (non-diabetic wt vs. diabetic wt: 51 ± 7 and 86 ± 19 PC/per mm2 of capillary area; $p < 0.001$). Therefore, the extent of increased pericyte migration in streptozotocin induced diabetic animals was comparable to that observed in spontaneous diabetic XLacZ mice. Overexpression of Ang-2 in non-diabetic retinas was sufficient to mimick retinal pericyte migration of diabetic wild- type litters with increased numbers of migrating pericytes of 78% (non-diabetic wt vs. non-diabetic mOpsinhAng2: 51 ± 7 and 90 ± 26 PC/per mm2 of capillary area; $p < 0.01$). Furthermore, overexpression of Ang-2 in diabetic mice increased the numbers of migrating pericytes by 2.3 fold compared with non-diabetic wild-type mice (non-diabetic wt vs. diabetic mOpsinhAng2: 51±7and118±25PC/per mm2 of capillary area; $p < 0.0001$) and by 37% compared with diabetic wild-type litters (diabetic wt vs. diabetic mOpsinhAng2: 86 ± 19 and 118 ± 25 PC/per mm2 of capillary area; $p < 0.05$). Quantitation of pericyte migration in non-diabetic Ang-2 deficient mice showed a significant reduction of 36% (non-diabetic wt vs. non-diabetic Ang2LacZ: 51 ± 7 and 37 ± 10 PC/per mm2 of capillary area; $p < 0.05$) compared with non-diabetic wild-type mice. Noteworthy, diabetic mice deficient in Ang-2 completely lacked hyperglycemia-induced increase in pericyte migration (diabetic wt vs. diabetic Ang2LacZ: 86 ± 19 vs. 52 ± 15 PC/per mm2 of capillary area; $p < 0.01$) compared with diabetic wild-type litters. Nevertheless, hyperglycemia slightly enhanced pericyte migration in Ang2LacZ mice (non-diabetic Ang2LacZ vs. diabetic Ang2LacZ: 37 ± 10 vs. 52 ± 15 PC/per mm2 of capillary area).

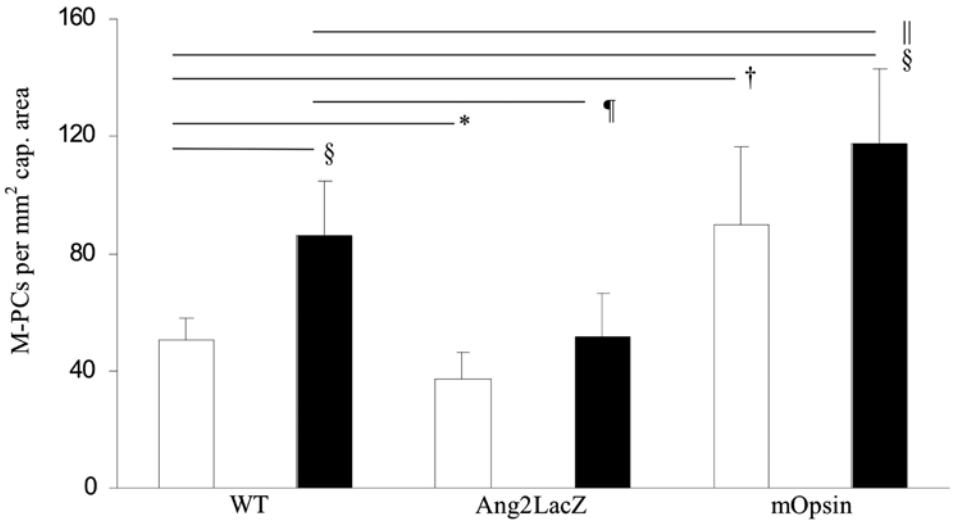

*Figure 5: Quantitation of migrating pericytes in non-diabetic and diabetic retinas with modulated Ang-2 expression (white bars: non-diabetic and black bars: diabetic). * $p < 0.05$, † $p < 0.01$ and § $p < 0.001$ compared with non-diabetic wild-type litters, ll $p < 0.05$, ¶ $p < 0.01$ compared with diabetic wild-type litters. WT: wild-type, mOpsin: overexpression of Ang-2 in mOpsinhAng2 mice, Ang2LacZ: downregulation of Ang-2 in Ang2LacZ mice*

Discussion

In this study, we describe a novel mechanism of pericyte loss in experimental diabetic retinopathy. We demonstrated that pericyte loss in diabetic retinopathy is restricted to a subset of pericytes located on straight capillaries. Hyperglycemia-induced loss of pericytes, predominantly on straight capillaries is accompanied by increased numbers of pericytes, migrating from same location into perivascular position. Increased numbers of migrating pericytes are LacZ negative. Furthermore, we show that Ang-2 is crucial for pericyte migration as Ang-2 deficient mice completely lack hyperglycemia-induced pericyte migration, whereas overexpression of Ang-2 mimics increased pericyte migration observed in diabetic retinas.

Experimental long-term hyperglycemia enhances pericyte detachment, leading to reduced pericyte coverage of retinal capillaries. Growing evidences suggest that pericyte detachment and migration from underlying vessels into the perivascular parenchym is a general feature of pericytes in response to different kinds of stress inducers. In brain capillaries, pericytes migrate from capillaries as a result of ischemia, hypoxia or injury [219, 220]. In response to traumatic brain injury, approximately 40 % of capillary pericytes migrated from their microvascular location and remained in a perivascular position, while remaining pericytes on capillaries displayed signs of degeneration. In tracheal capillaries, blockage of VEGF signaling resulted in migration of pericytes from the regressing capillaries onto surviving vessels, leaving behind empty basement membrane tubes, comparable to those observed in DR [221]. These data show that pericyte attachment is actively modulated depending on environmental conditions.

In the diabetic retina, pericyte loss is one of the earliest and most characteristic morphological changes. Our previous data revealed the importance of the Ang-2 / Tie-2 system in the regulation of vascular morphological changes in the diabetic retina. In diabetic rat retina, Ang-2 is upregulated before pericyte loss becomes evident and increased expression of Ang-2 correlates with the depletion of perivascular cells [33, 35]. Intravitreal injection of Ang-2 results in pericyte dropout and Ang-2 haploinsufficiency appears to protect against diabetes-induced pericyte dropout. Now, we show that Ang-2 regulates pericyte migration in experimental DR. In loss of function experiments we show that Ang-2 is required for hyperglycemia-induced migration of pericytes, as Ang-2 deficient mice lack increased migration of pericytes compared to diabetic wt mice. In addition, gain of function experiments showed that overexpression of Ang-2 enhances pericyte migration

in non-diabetic and in diabetic animals. The extent of pericyte migration, induced by Ang-2 overexpression in non-diabetic retinas was similar to the one observed in wild-type diabetic animals, highlighting the importance of the Ang-Tie system in cellular crosstalk and its involvement in earliest stages of diabetic retinopathy. Nevertheless, we found a trend for a hyperglycemia-induced increase in pericyte migration in Ang-2 overexpressing and deficient retinas, suggesting that other factors than Ang-2 may be of some importance for pericyte migration. Recent observations in microvascular endothelial cells revealed that high glucose increases the expression of Ang-2 through modification of corepressor mSim3A by methylglyoxal, an important intracellular AGE [72]. As methyglyoxal is the predominant intracellular AGE in the diabetic retina this study links biochemical changes of diabetes to increased activation of Ang-2/Tie-2 system and therefore to our observation, that hyperglycemia enhance detachment and migration of pericytes from microvasculature, resulting in typical pericyte loss of DR.

Moreover, our data demonstrate that hyperglycemia-induced loss of pericytes is not equal in retinal pericyte subpopulations, such as it is restricted to pericytes located on straight capillaries of the retinal microvasculature. Straight capillaries are the only sites from which pericytes migrate. According to our observations, hyperglycemia reduces the number of S-PCs by approximately 460 cells per mm2 of capillary area after six months of hyperglycemia and simultaneously increases the numbers of migrating pericytes by about 80 cells per mm2 of capillary area. The gap between the numbers of lost and migrating pericytes is partly explained by the digestion process as this eliminates pericytes which are completely dissociated. Furthermore the retinal digest preparations represent snapshots of the permanently remodeling vasculatures. To clarify the absolute contribution of pericyte migration to pericyte loss in experimental diabetic retinopathy and the destiny of completely dissociated pericytes needs further investigations.

LacZ in XLacZ mice labels about 52% of all pericytes and is therefore a restricted pericyte marker, such as others. Nevertheless, quantitation of total pericyte numbers and the expression of LacZ showed that only LacZ positive pericytes are significantly reduced in diabetic retinas. Therefore, quantification of LacZ expressing pericytes may provide a simplified method for the quantification of pericyte loss in experimental diabetic retinopathy. Furthermore, we showed that increased numbers of migrating pericytes rise from pericytes which are LacZ negative. This is in agreement with published data showing that proliferation and migration of LacZ expressing cells are associated with transgene

down-regulation and vessel injury leads to changes in marker expression in pericyte subpopulations [169].

The causes of early pericyte loss in DR have not been fully delineated. Numerous studies demonstrated that increased levels of glucose and AGEs trigger pericyte death in cell culture and that pericyte apoptosis is increased in human diabetic retinas as well as in experimental DR [97, 222-224]. However, pericyte apoptosis fails to explain total extent of pericyte loss and its time course in experimental DR. In animal models of DR, pericyte apoptosis has been detected at later stages, after more than six months of hyperglycemia [117]. Not compatible with this observation is, that significant pericyte loss is already detectable after three months of experimental diabetes [28]. Pericyte migration, as an alternative or additional mechanism of pericyte loss in diabetic retinopathy offers a possible explanation for the discrepancy between total extent of pericyte loss and published data of pericyte apoptosis in the diabetic retina.

In summary, our data provide morphological evidence that pericyte migration represents a novel mechanism of pericyte loss in the diabetic retina. We further show that this mechanism is regulated by signaling via the Ang-2/Tie-2 pathway. The exact mechanism underlying this process and the destiny of resting and migrating pericytes remain to be investigated.

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